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THE REGULATION OF CHONDROCYTE DIFFERENTIATION BY RHO GTPASES AND THE ACTIN CYTOSKELETON

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Purpose: There are likely many parallels of genes that regulate chondrocyte differentiation in development and pathology in osteoarthritis. We have identified the role of Rho signaling and the actin cytoskeleton as important regulators of chondrocyte differentiation in development. We have shown that RhoA/ROCK signaling is inhibitory to early stages of chondrocyte differentiation, at the level of suppression of Sox9 transcription. Furthermore, we have identified Rac1 and Cdc42 as promoters of chondrocyte differentiation at the level of regulating the cell adhesion molecule, N-Cadherin and Sox9 transcription, respectively. In parallel to these studies, we have shown that inhibition of actin polymerization by cytochalasin D and promotion of actin polymerization by jasplakinolide promotes chondrogenesis at the level of promoting Sox9 transcription.
Methods: We have extended these studies to the later stages of chondrocyte differentiation to hypertrophy. Using high density monolayer cultures of chondrocytes isolated from embryonic growth plates, we treated cells for 24 hours with the ROCK inhibitor Y27632, cytochalasin D or jasplakinolide, isolated RNA, hybridized to Affymetrix 430 MOE chips and analyzed by microarray. Genes that show at least a two fold change are compared to microarray data obtained in our lab of microdissected embryonic tibia. Common genes are chosen for further studies, by first confirming fold changes by realtime PCR. Normal expression patterns of selected genes are analyzed in embryonic tibia growth plates and compared to growth plates treated with the aforementioned inhibitors.
Results: Our data demonstrate that the majority of genes that are upregulated by cytochalasin D treatment are also upregulated in the hypertrophic region of the growth plate. Functional studies are currently being completed on novel genes that are significantly changed in both hypertrophy and inhibitor treatment. Rorα and FRZB are upregulated in hypertrophy and by cytochalasin D. GDF10 is upregulated by Cytochalasin D, Y27632 treatment and in hypertrophy. Osteomodulin is downregulated by Cytochalasin D, upregulated by Y27632 and hypertrophy. L-plastin is commonly upregulated by all three inhibitors and hypertrophy.
Conclusions: In order to identify targets for osteoarthritis, we need to develop a more conmprehensive understanding of chondrocyte differentiation in development. Rho signaling and it's targets may prove to be quite important in modifying pathologies such as osteoarthritis.

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PERICELLULAR MATRIX MODULATES HEAT SHOCK PROTEIN EXPRESSION BY MECHANICALLY STIMULATED HUMAN CHONDROCYTES

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Purpose: The pathophysiology of osteoarthritis (OA) involves an imbalance between anabolic and catabolic pathways leading to progressive degeneration of the cartilage matrix. In addition to their roles in protein folding, stabilization and transport, heat shock proteins (HSPs) protect cells from cytotoxic effects of stresses such as inflammatory cytokines and nitric oxide. Expression levels of HSPs have been related to OA severity, and gene transfer of HSPs confers cytoprotection in experimental OA. The pericellular matrix (PCM) modulates the mechanical responsiveness of chondrocytes and we have recently reported that retention of the native PCM increases HSP expression in isolated chondrocytes. In this study we investigated the role of the PCM in regulation of HSP expression in response to mechanical stimulation.
Methods: Human articular cartilage was obtained as waste tissue from total joint arthroplasties with approval from the UNC IRB. Chondrocytes and chondrons (chondroyctes with PCM intact) were isolated enzymatically and maintained in alginate bead culture for up to one week before subjecting a subset of each culture from individual specimens to heat shock (1 hr. at 42°C) or cyclic hydrostatic pressure (cHP; 7.5 MPa, 1hz, 4 hours). Matched control samples of each culture were held at 37°C for the duration of treatment. After treatment, cells were recovered from alginate and RNA was isolated and analyzed by real-time rtPCR for expression of HSP 27, HSP 70, HSP 90 and caspase 3 as well as GAPDH for normalization.
Results: There were notable differences between chondrocytes and chondrons in response to both heat shock and cHP. After heat shock expression levels of the three HSPs increased above baseline in both chondrocytes and chondrons ($p<0.01$), but chondrocytes had approximately 2-fold greater activation of HSP 70 and HSP 90 than chondrons (p=0.03, 0.01, resp), while chondrons had 2-fold higher activation of HSP 27 (p=0.02). In response to cHP, HSP 27 did not change significantly in any cultures. HSP 70 was significantly elevated above baseline in chondrocytes (p=0.02) but not chondrons, while HSP 90 expression was elevated in chondrons (p=0.04). There were no significant changes in caspase 3 expression in chondrocytes or chondrons following heat shock or cHP, supporting the conclusion that the stresses did not induce apoptosis.
Conclusions: We have shown that the presence of the native PCM modifies the response of chondrocytes to stress, including mechanical stimulation. We have further demonstrated that primary OA chondrocytes are baro-responsive. Previous reports have suggested that while primary chondrocytes are non-responsive to HP, early changes in OA may cause chondrocytes to lose baro-resistance. HSP 70 and 90, differentially regulated by chondrons and chondrocytes, interact with signaling pathways including caspase 3 and the NF-kB pathway. Our data suggest that the PCM is an important regulator of mechanically induced cell signaling, and alterations to the PCM may affect the progression of OA.

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FUNCTIONAL TESTING OF DIARTHRODIAL JOINT SOFT TISSUES WITH A ROLLING-PLOWING APPARATUS: VALIDATION AND FIRST RESULTS

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Purpose: Dynamic stereometry of the TMJ, i.e. a software reconstruction of real anatomy animated by its real kinematics, combined with numerical modeling can provide in vivo data on strains, forces, stresses, and work density, that improve understanding of craniomandibular biomechanics and are needed for successful biomimetics. Earlier we showed that plowing effects may occur in the TMJ fibrocartilaginous disc, so that this might get damaged being weaker in mediolateral direction. However, the biological response to this complex mechanical environment is still unclear. Therefore, a larger project has been started in order to reproduce the rolling-plowing as recorded in the TMJ (and other diarthrodial joints) on live cartilage explants and analyze the biological reaction to this type of dynamic loading. This presentation will illustrate the construction and validation of

the rolling-plowing testing system and first results on live tissue specimen.

Methods: An apparatus was developed consisting of an arm moving in the horizontal plane by means of linear stages. The arm holds a custom-milled indenter rotated by a micromotor and pushed vertically by a linear actuator against the cartilage specimen. This is kept in a tank filled with DMEM solution. Coordinated computer motor control of adequately milled indenters reproduces subject-specific stress-field paths and instantaneous plowing aspect ratios measured across the TMJ disc tissue during mandibular movement. Load cells and LVDTs measure force and displacement in x, y and z direction. Validation of the system was performed by dynamic stereometry on a toroidal indenter moving linearly and cyclically along 100 mm and pushed with a normal force of 5-40 N onto bovine nasal septum strips of 120x15x3 mm glued on plexyglas. Furthermore, cell viability was assessed by means of the FDA/PI Dye Inclusion and Exclusion Assay after static and dynamic loading. Static loading was performed with maximum contact pressures of 0.15-0.5 MPa and maximum shear stresses of 0.05-0.15 MPa for 2, 4, 6, and 10 min. Dynamic loading was performed with a normal force of 10-100 N for periods of 30-300 min while plowing the tissue cyclically over an excursion of 10 mm with a sinusoidal velocity profile (f=1 Hz, vmax=40 mm/s).

Results: Stress-field translation velocity profiles of up to 100 mm/s could be reproduced with accuracy better than 1.5%. For the different normal forces, a contact area wide from 4 to 8 mm was measured. A linear relationship ($R2 > 0.86$) was found between contact area widths and number of points needed to define the contact area (n = 5 to 40) in the stereometry model. A mesh with a mean polygon area of 2.7 mm2 showed the best correspondence between measured trace widths and the values calculated in the model. At all static loads the surface of the cartilage remained intact and all chondrocytes were viable. After dynamic loading, mechanical damage and also cell death started to occur above a combined threshold of load and duration (e.g. 90 N & 45 min, 70 N & 60 min, 60 N & 240 min). In these specimens, cell death started from the face of the cartilage strip opposite to the side where the cylinder had been plowing. Overall, the manipulation of the cartilage strips in order to obtain precise geometric shapes did not affect the viability of the chondrocytes.

Conclusions: This study shows the validity of a novel rolling-plowing apparatus for functional testing of live cartilage tissue of diarthrodial joints. Contact areas can be indirectly determined by graphic computer modeling. The mesh density appears to be crucial and needs to be optimized for a correct estimation of the contact areas. Bovine nasal septum can be a convenient model to study chondrocyte mechanobiological response to complex loading patterns.

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PLASMID BASED RNA INTERFERENCE CONTROLS INTERLEUKIN-1 EXPRESSION IN OSTEOARTHRITIS

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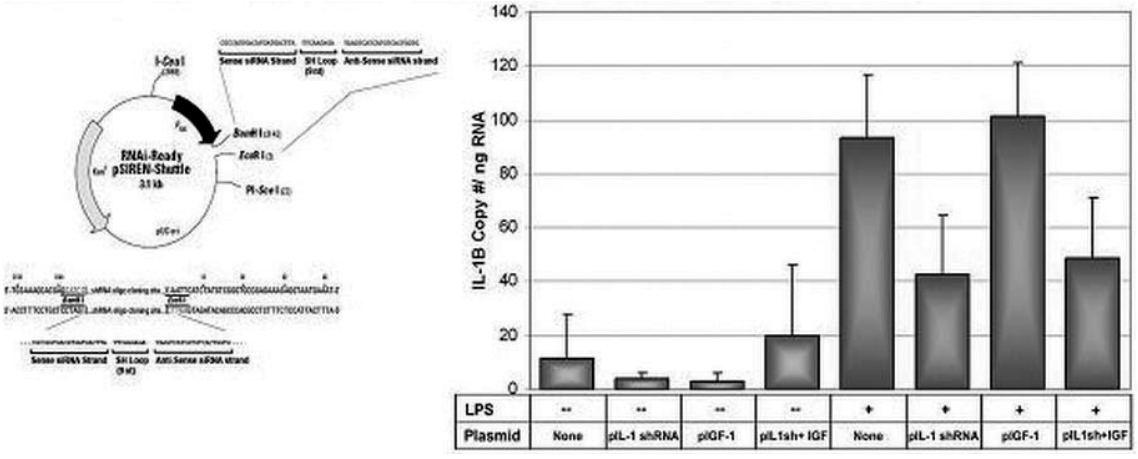
Purpose: Osteoarthritis is characterized by an imbalance in cartilage catabolic and anabolic processes. Combinatorial therapies may enhance pain relief and improve cartilage function through knockdown of IL-1 and supplementation of growth factors such as IGF-I. Small interfering RNA (siRNA) molecules bind to mRNA to silence specific target genes post-transcriptionally. The hypothesis for this study was that cognate IL-1 gene expression could be diminished by RNA interference, which would quell degradatory cycles in the osteoarthritic joint and allow IGF-I gene enhanced repair.

Methods: Small interfering RNA's (siRNA) targeting IL-1B were synthesized and screened by electroporation in chondrocyte monolayers exposed to LPS. Gene expression for IL-1beta and IL-1alpha and MMP-13 were returned to baseline by several IL-1 silencing ribo-oligo's. IGF-I and cartilage matrix molecules remained supressed after LPS insult of monolayer cultures, despite IL-1 silencing. Effective siRNA ribo-oligo's were constructed as plasmid based short hair-pin coding constructs to prolong IL-1 knockdown (pSIREN-IL-1shRNA), utilizing a pol III promoter and activation by Dicer processing. Additional plasmid vectors were constructed containing the CMV promoter and IGF-I gene, and a combination of IGF and IL-1β shRNA coding regions, resulting in a bicistronic plasmid expressing IGF-I mRNA, and the IL-1β silencing motif. The most efficient plasmid systems for shRNA formation and growth factor (IGF-I) gene enhanced cell function were re-ligated into transposon based Sleeping Beauty vectors, and evaluated in chondrocytes and synoviocytes.

Results: Plasmid based delivery of IL-1 short hairpin loops with or without IGF-I gene transduction prolonged the matrix restorative effects. Chondrocyte monolayers electroporated and selected by puromycin exposure, were actively expressing IL-1shRNA and had $> 60\%$ reduction in IL-1 abundance (Fig. 1). Stable robust IGF-I over-expression was resistant to IL-1 induced non-responsiveness (Fig. 2). These data confirm that IL-1 RNA interference can be conveyed by plasmid based shRNA syn



Abstract 193 – Figure 1. Left: IL-1shRNA plasmid map showing coding region for IL-1shRNA loop. Right: IL-1β expression in chondrocytes conditioned by LPS exposure (LPS) and transduced with pSIREN-IL-1shRNA (pIL-1), pIRES-puro3-IGF (pIGF), or pIRES-puro3-IGF-IL-1shRNA (pIL1sh+IGF). LPS induced a robust IL-1 response, which was significantly reduced by IL-1 RNA interference (pIL-1shRNA or pIL-1sh+IGF). Chondrocytes transduced with pIGF continued to express elevated IL-1 mRNA under the influence of LPS.